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DT[redacted]oc'd PCT/PTO 14 JUL 2004

European Patent Office
International Preliminary
Examining Authority
D-80298 Munich
ALEMANIA

10/501364

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MIT/PCT-127

29 April 2004

FAX Nº 00 49 89 2399 4465

CONFIRMATION BY COURIER

Re: International Patent Application No. PCT/ES03/00063
VITATENE, S.A., et al.

Reply to written opinion (WO) pursuant PCT Rule 66

We reply in due time to the WO drafted by the EPO actins as IPEA in PCT IPE proceedings.

We enclose hereto an amended set of claims in reply to the objections raised in the above mention opinion

* Support for the amendments made

i. Amended claims 1-5 cover the method of producing astaxanthin overproducing strains of *X. dendrorhous* and they are supported in the description at: page 8, line 34 to page 10, line 28; page 9 line 29 to pg. 10 line 28; scheme 2 at page 15.

ii. Amended claims 6 and 7 cover the mutants obtained thereof and they are both supported in the description at page 10 line 29 to page 11 line 1; example 6.1.; former claims 1 and 2 (partially).

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iii. Amended claims 8-23 cover the fermentation process for producing astaxanthin using said overproducing mutants in different culture conditions. Those claims correspond approximately to formerly filed claims 1 to 22.

iv. Amended claims 24-27 cover the biomass obtained after the fermentation and the compounds consisting or containing the same. Those claims correspond precisely to former claims 23-26.

* Re Item IV: Lack of unity

The amended set of claims covers a single underlying inventive concept comprising:

a) Obtaining new astaxanthin overproducing mutants of *X dendrorhous* consisting in the process of mutation-selection and the mutants obtained therein.
b) A fermentation process for producing astaxanthin based on those mutants.
c) The biomass obtained throughout that fermentation process, which includes the mutants and the astaxanthin produced therein and any compound which consists or contains the aforesaid biomass.

In our view the general inventive concept covered by the amended set of claims it would fulfil the unity requirement according to Rule 13.1. PCT. The single inventive concept to be addressed as discussed later on, it would consist in finding an alternative process for producing astaxanthin with high yield.

* Re Item V.2.: Clarity

All the former claims (1, 2, 12 and 19-22) previously defined only by the minimum yield (concentration) of astaxanthin or biomass to be achieved have been either removed or amended to further define them by structural essential features (see, for example, amended claim 6 in that respect).

* Re Item V.3.: Novelty

None of prior art documents D1-D8 cited in the WO discloses a method of obtaining mutants of *X dendrorhous* or *P. rhodozyma* which uses the initial selection step of growing the mutants on a medium containing inhibitors of the synthesis of steroids, or compounds that alter yeast cell redox potential by, mainly, inducing formation of free radicals; followed that initial selection step by further selection steps based on any of the technical features (i-iv), as now claimed in amended main independent claim 1 and amended dependent claims 2-5. Hence, amended claims 1-5 are rendered novel. The mutants obtained through that new process, in view of its genomic pattern which confers to them new technical features linked to astaxanthin production, they are also rendered novel over the cited prior art. None among D1-D8 discloses mutants having extrachromosomal elements formed by double stranded DNA plasmids. The fermentation process covered by independent claim 8, which uses as

fermentation inoculum and vegetative fermentative biomass the new mutants is consequently novel too. The process variants as claimed in dependent claims 9-23 should also be acknowledged as new in view of its dependency on independent claim 8. Moreover, claims 24-27 which cover the outcome of the fermentation process as biomass product, which includes the new yeast mutants, or any compound consisting or containing said biomass, should also be recognized as novel.

We notice the statement made by the examiner about the novelty recognition in respect of former claims: 5-12 and 17-22 (see 3.2^{c} and 3.4).

* Re Item V.3.: Inventive step

We notice the statement made by the examiner recognizing the inventive step of former claims 5-12 and 17.

The objective technical problem to be solved with the amended set of claims is to find an alternative process for astaxanthin production based on new mutants.

Closest prior art documents may be either D1, D2, D3 or D4 which they all screen astaxanthin overproducing mutants of *Phaffia rhodozyma* by growing the strains mutated in solid medium and by selecting colonies more coloured than the parent strain (see D1, pg 9, lines 28-33; D2, pg.3 lines 1-2; D3 –Spanish version-, pg. 3, lines 31-34; D4, pg.3, lines 9-12). The state of the art suggests looking for mutants which overproduce the pigment astaxanthin in standard conditions. As matter of fact there are in that state of the art, documents which show fermentation yields even higher than the ones achieved with the mutants obtained in the invention (see D1 for fermentation processes rendering yields over 5000 ppm). Standard conditions of culture should be understood as, among other: using glucose as carbon source (see D1, pg. 10, line 23), at reduced temperatures (see description page 10, lines 15-17; D1, pg. 9, line 21; D3 -Spanish version-, pg. 3, line 60), under light conditions (see description pg. 11, lines 24-25; and items 1.1^{a}, 1.3^{a} and 1.6 of the WO with reference to D1, D4 and D6) and without inhibitory compounds for carotenoid synthesis or compounds inducing the formation of free radicals, hence altering the yeast cell redox potential. The solution found and now claimed in the invention follows a different approach. It intends to find out mutants that, while maintaining a high yield astaxanthin production, they were also able to grow in presence of compounds with inhibitory activity against steroid synthesis. That is important because astaxanthin and carotenoids share many biosynthetic pathway steps in common (see scheme 1 at page 5). By adding inhibitors of the steroid synthesis in the growing medium the following possibilities may arise:

a) Many strains would not grow up at all
b) Many of them would posses only a slight red colour
c) Some of them would develop a deeper red colour and that means they will overproduce astaxanthin, because the red colour due to the production of other steroid is inhibited by the presence in the medium of steroid synthesis inhibitory compounds.

In absence of steroid synthesis inhibitors, the red colour of the mutant colonies obtained, is so deep that it is almost impossible to select deeper colour colonies. The colour may be due either to astaxanthin syntheis or to other carotenoids synthesis, making impossible to select mutants exhibiting astaxanthin overproduction by simply determination of the colour and without a further growing o fermentation to determine whether or not astaxanthin is produced by each mutant forming in solid medium coloured colonies. Unexpectedly, the mutants surviving the initial selection made in present invention, by culturing them in presence of steroid synthesis inhibitors overproduce astaxanthin. The same occurs, surprisingly when that initial selection is made in presence, instead of steroid synthesis inhibitors, in presence of compounds that induce the formation of free radical into the yeast cell altering its redox potential. An analogous approach was disclosed in D4 (pg. 2, lines 54-56). D4 achieves the inhibition of cell respiration by means of inhibiting the electron transport chain. However, in D4 those inhibitors, that have not been used in the present invention, were used in substitution of the mutation agents and, contrary to what happens in our application, did not produce stable biological material.

The combination of that first selection step with any of the other selection routes is not neither trivial at all, nor obvious for a man skilled in the art. With the first selection step astaxanthin overproducing mutants were selected. Additionally those mutants would posses (see description pg.8, lines 2-3) low levels of accumulation of other carotenoids. Further selecting mutants with ability to grow by using sucrose as carbon source, being glucose an expensive carbon source, renders the fermentation process much cheaper. Selecting mutants able to grow at higher temperatures (24 °C) when maximal production of astaxanthin is obtained by fermentation in normal conditions by cultivating the strains at 17-20, 21°C, avoids any refrigeration means to be applied to the cubes of fermentation, especially during the summer and particularly in sunny and hot weather countries. By using the astaxanthin overproducing mutants obtained in the invention capable of producing astaxanthin at higher temperatures, those refrigeration means are no longer necessary. The same happens with the mutants with ability to produce astaxanthin in darkness. Optimal production of astaxanthin by fermentation is achieved in the presence of light and that means that the fermentation cubes which use to be structurally closed, need to be supplied inside with illumination means that, apart that the may rise the temperature fermentation decreasing hence astaxanthin yield, they make the installation also more expensive and complicated. Therefore, the mutants obtained and selected by the invention solve all those problems existing in the prior art and that D1-D8 intend to solve by different routes or even do not foresee them at all. Hence amended claims should be recognized as having inventive step over the cited prior art.

Notwithstanding previous statements about the novelty and inventive step of the invention, this representative request under Rule 66.6 PCT to maintain any informal communications as the examiner may find necessary to solve any remaining objection or as a matter of clarification of any aspect related to this reply to the WO. We also respectfully request under Rule 66.4 PCT to have an additional opportunity to submit further amendments, if the

present reply to the WO would not overcome yet all the objections raised therein or it would raise some new matters of concern.

Yours faithfully,

ELZABURU

Manuel Illescas

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